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Spontaneous DNA Synthesis by Subpopulations of Lymphocytes in Hodgkin's Disease*

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Abstract—*After preselection on the basis of specific gravity, peripheral blood lymphocytes from ten patients with Hodgkin's disease were separated into an E, an EAC and a non-E/non-EAC subpopulation by rosette sedimentation techniques. The spontaneous DNA synthesis of these subsets was quantified by ^{3}H-thymidine incorporation, measured by scintillation counting. The highest spontaneous proliferation was found in the non-E/non-EAC subgroup in comparison to the E* ($P<0.01$) *and the EAC subpopulation* ($P<0.001$).

INTRODUCTION

ANTIGENIC and mitogenic stimulation of human lymphocytes *in vitro* causes proliferation detectable as DNA synthesis by means of ^{3}H-thymidine incorporation. With this technique, Crowther *et al.* [1] demonstrated the presence of activated lymphocytes in the peripheral blood of patients with Hodgkin's disease. At the time of their study techniques of identifying human B and T cells were not yet available. Huber *et al.* [2] autoradiographically showed that proliferating cells were preferentially T lymphocytes, although they also found an augmented ^{3}H-thymidine uptake by the non-T cells of patients with Hodgkin's disease in comparison to normal controls. In the present study we examined in Hodgkin's disease patients the DNA synthesis in the various lymphocyte subsets during short term unstimulated cultures. DNA synthesis was quantitated by ^{3}H-thymidine incorporation, measured by liquid scintillation counting. As the majority of spontaneously stimulated blood lymphocytes have a relatively low specific gravity [3] enrichment of low density lymphocytes was obtained by gradient centrifugation before lymphocytes were separated by rosette sedimentation.

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MATERIALS AND METHODS

Patients

Ten consecutive newly diagnosed and untreated patients with Hodgkin's disease were included in this study. There were no signs of overt infection at the time of investigation. Clinical data are summarized in Table 1. The histological material was classified as described by Lukes *et al.* [4]. Staging procedures were carried out according to the criteria recommended at the Ann Arbor Conference [5].

Isolation and culturing of lymphocytes

Lymphocytes were isolated from peripheral blood by a method described earlier [6]. Briefly, monocytes and granulocytes were removed from defibrinated blood by nylon wool filtration at a constant flow rate of 1.4 ml/min. The filtrate was depleted from erythrocytes by density centrifugation. By this technique an average lymphocyte purity of 96% is achieved, whereas the contamination by monocytes varies from 0 to 1.6%. The obtained lymphocyte suspension was divided into a fraction with a relatively low and a relatively high density by means of a Ficoll (mol. wt 400,000; Sigma Chemical Company, St. Louis, U.S.A.) Isopaque (440; Nyegaard and Co. A.S., Oslo, Norway) discontinuous gradient with a S.G. of 1.068 g/ml. After centrifugation at 1000 ***g*** for 20 min at 4°C the interphase containing the low density lymphocytes was collected and washed with Tris buffered minimal essential medium (M.E.M., Gibco,

Grand Island Biological Company, Grand Island, New York, U.S.A.) at 600 *g* for 20 min at room temperature. After determining the percentage of sheep red blood cells (SRBC) rosette forming lymphocytes (E-cells), these low density lymphocytes were distributed into a E and non-E population according to Pellegrino *et al.* [7]. The non-E lymphocytes, after washing with Tris buffered MEM, were incubated with SRBC coated with antibody–complement complexes (EAC-cells), as reported by Bianco *et al.* [8].

This suspension was gently resuspended, layered over Ficoll–Isopaque and spun at 1000 *g* for 30 min at room temperature. The non-EAC rosetting lymphocytes were recovered from the interphase and the EAC rosetting cells from the pellet. After washing with Tris buffered MEM, the E, EAC and non-E/non-EAC suspensions were incubated for 30 min at 37°C with autologous complement in order to lyze the SRBC.

Afterwards the various lymphocyte suspensions were washed with Tris buffered MEM. The cell concentrations were adjusted to 3×10^5/ml [9] by adding Tris buffered MEM containing 20% inactivated pooled human serum, and supplemented with penicillin (100 I.U./ml) and streptomycin (100 μg/ml). Aliquots of 1 ml from each suspension were cultured without addition of any stimulant, after adding 0.25 μCi ^{3}H-thymidine (Radiochemical Centre, Amersham, England; sp. act. 5 Ci/mmole) in 0.1 ml MEM. After 24 hr. the cells were harvested on Millipore® microfiber glassfilters. The ^{3}H-thymidine uptake was measured in counts/min with a liquid scintillation counter. All cultures were carried out in triplicate under sterile conditions.

Detection of surface immunoglobulin

The presence of surface immunoglobulins on the non-E/non-EAC lymphocytes were evaluated according to van Oers *et al.* [10].

Statistics

The results of the ^{3}H-thymidine incorporation studies were compared by means of Wilcoxon's Sign Rank Test.

RESULTS

The results of the spontaneous ^{3}H-thymidine incorporation into various low density lymphocyte subpopulations are shown in Table 1.

Uptake by the non-E/non-EAC cells (mean 940, range 350–3200 counts/min) was significantly higher than the uptake by the E-cells (mean 437, range 240–880 counts/min; $P < 0.01$) and the uptake by the EAC-lymphocytes (mean 210, range 120–390 counts/min; $P < 0.001$). Furthermore, the values measured in the E-cells were higher than in the EAC-cells ($P < 0.001$).

The mean percentage of the low density lymphocytes forming E rosettes was 57% (range 32–76; $n = 10$). After E rosetting sedimentation more than 95% E rosette positive lymphocytes were found in the pellet, whereas the interphase contained less than 1% E rosette positive cells ($n = 6$). The mean percentage of the non-E lymphocytes forming EAC-rosettes was 65% (range 40–80; $n = 10$).

Table 1. Some clinical data of the patients and spontaneous ^{3}H-thymidine incorporation into the various low density lymphocyte subpopulations

Subject	Sex	Age	Histology	Stage	^{3}H-Thymidine incorporation (counts/min)			
					Unseparated lymphocytes	E cells	EAC cells	non-E/non-EAC cells
1.	M	38	N.S.	PS IIIA	300	320	130	610
2.	F	43	N.S.	CS IIIB	2250	240	145	3200
3.	M	25	N.S.	PS IIA	1030	455	225	1155
4.	M	27	M.C.	CS IVB	755	630	390	625
5.	F	22	N.S.	PS IA	615	275	205	405
6.	F	19	N.S.	PS IA	555	320	190	350
7.	F	22	L.P.	PS IIIA	350	445	120	585
8.	M	57	M.C.	CS IVA	630	880	235	885
9.	M	18	N.S.	CS IVB	515	435	230	1000
10.	M	40	M.C.	CS IVA	415	370	225	580

M = male; F = female; N.S. = nodular sclerosis; M.C. = mixed cellularity; L.P. = lymphocyte predominance; P.S. = pathological staging; C.S. = clinical staging.

Centrifugation after EAC-rosette formation revealed more than 90% EAC positive lymphocytes in the pellet, and less than 3% in the interphase ($n=6$) among the non-E/non-EAC lymphocytes.

The presence of surface immunoglobulin was evaluated in six experiments. We detected an average of 3.5% positive cells (range 1–7).

DISCUSSION

Increased spontaneous DNA synthesis by lymphocytes in patients with Hodgkin's disease is well documented [1, 11].

The results of the present study show preferential proliferation of surface immunoglobulin negative non-E/non-EAC lymphocytes in comparison to the E and EAC subpopulations. The non-E/non-EAC subpopulation is the least defined amongst the various lymphocyte subgroups. It comprises probably functionally distinct cell types [12, 13].

Huber *et al.* [2] found that the major fraction of DNA synthesizing blood lymphocytes belonged to the E-cell series in comparing E with non-E lymphocytes. They used autoradiography and did not discrimate between high and low density lymphocytes or between EAC and non-E/non-EAC cells. So, it is possible that the activity of the non-E/non-EAC was concealed by by a majority of inactive EAC lymphocytes present in the same non-E cell subset.

Ehrnst *et al.* [14] observed an increasing number of non-E/non-EAC lymphocytes during the peak of DNA synthesizing acticity after vaccination against yellow fever. Moreover, they found the highest values of ^{3}H-thymidine incorporation within the non-E/non-EAC population. In contrast, the basic spontaneous blastogenesis of peripheral lymphocytes in normal subjects seems to be located in the E-cells [15].

Whereas spontaneous DNA synthesizing lymphocytes in Hodgkin's disease as well as after vaccination preferentially are non-E/non-EAC cells, it is tempting to assume an analogous origin and activation. Our findings and those of Huber *et al.* [2] may point to a multicellular involvement in the spontaneous lymphocyte transformation in Hodgkin's disease.

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